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Secoisolariciresinol and isotaxiresinol inhibit tumor necrosis factor-α-dependent hepatic apoptosis in mice

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Abstract

The effects of secoisolariciresinol (**1**) and isotaxiresinol (**2**), two major lignans isolated from the wood of *Taxus yunnanensis*, on tumor necrosis factor-α (TNF-α)-dependent hepatic apoptosis induced by D-galactosamine (D-GalN)/lipopolysaccharide (LPS) were investigated in mice. Co-administration of D-GalN (700 mg/kg) and LPS (10 μg/kg) resulted in a typical hepatic apoptosis characterized by DNA fragmentation and the formation of apoptotic bodies. Serum glutamic pyruvic transaminase (sGPT) and glutamic oxaloacetic transaminase (sGOT) levels were also raised at 8 h after D-GalN/LPS intoxication due to a severe necrosis of hepatocytes. Pre-administration of **1** or **2** (50, 10 mg/kg, *i.p.*) 12 and 1 h before D-GalN/LPS significantly reduced DNA fragmentation and prevented chromatin condensation, apoptotic body formation and hepatitis. Pro-inflammatory cytokines such as TNF-α and interferon-γ (IFN-γ) secreted from LPS-activated macrophages are important mediators of hepatocyte apoptosis in this model. Pre-treatment with **1** or **2** significantly inhibited the elevation of serum TNF-α and IFN-γ levels. In a separate experiment, both lignans had a significant dose-dependent protective effect on D-GalN/TNF-α-induced cell death in primary cultured mouse hepatocytes and TNF-α-mediated cell death in murine L929 fibrosarcoma cells. These results indicated that **1** and **2** prevent D-GalN/LPS-induced hepatic injury by inhibiting hepatocyte apoptosis through the

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blocking of TNF-α and IFN-γ production by activated macrophages and direct inhibition of the apoptosis induced by TNF-α.

Keywords: Secoisolariciresinol; Isotaxiresinol; Apoptosis; *Taxus yunnanensis*; Hepatoprotective effect; TNF-α; D-Galactosamine; Lipopolysaccharide; Lignan

Introduction

Lignans are found in a variety of foods, vegetables and fruits that have potential protective effects on human health. Interest in this class of compounds is increasing because of their possible function as weak estrogenous or estrogen antagonists (Nicolle et al., 2002). Enterolactone and enterodiol, the two main lignans identified in human urine and plasma, are considered to derive from colonic bacterial metabolism of the plant-derived precursors matairesinol and secoisolariciresinol (**1**), respectively (Axelson et al., 1982; Borriello et al., 1985). Both experimental and epidemiological studies suggest that high plasma and urinary concentrations of phytoesterogens, including lignans, are associated with a decreased risk for hormone-dependent diseases e.g., breast cancer and coronary heart diseases (Vanharanta et al., 1999; Ingram et al., 1997). Consequently, research has been done on secoisolariciresinol diglucoside (SDG), a potent source of mammalian lignans, found in flaxseed (Ford et al., 2001). SDG is a cancer chemopreventive agent effective against the onset of breast, prostate and colon cancers; additionally it also displays antioxidant activity, and exhibits an ability to reduce the development of streptozotocin-induced diabetes (Thompson et al., 1996; Kitts et al., 1999; Prasad, 2000). Secoisolariciresinol (**1**), the aglycone of SDG, on the other hand, is not well studied in terms of its pharmacological properties even though it is formed as an intermediate during the processing of plant lignans by anaerobic fecal micro flora in mammals and also detected in the blood of rats fed phytoesterogens (Nicolle et al., 2001; Axelson et al., 1982; Borriello et al. 1985; Heinonen et al., 2001). Recently, we isolated secoisolariciresinol (**1**), a simple dibenzylbutane lignan (Fig. 1) from the wood of *T. yunnenensis*, together with isotaxiresinol (**2**), a closely related lignan having an aryltetralin skeleton (Banskota et al., 2002). Both of these compounds possessed potent 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and significant inhibitory activity against nitric oxide (NO) production in lipopolysaccharide (LPS)-activated murine macrophage-like

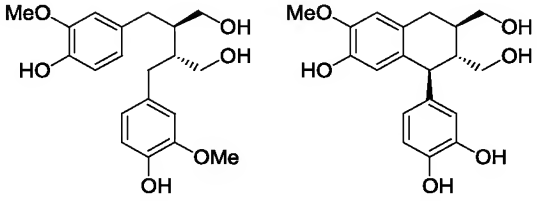

Fig. 1. Structure of secoisolarisiresinol (**1**) and isotaxiresinol (**2**) isolated from the wood of *T. yunnanensis*.

J774.1 cells (Banskota et al., 2003). Moreover, secoisolariciresinol (**1**) also possessed antiproliferative activity against human HT-1080 fibrosarcoma cells (Banskota et al., 2002). In order to explore their pharmacological properties, we further investigated the effect of **1** and **2** on tumor necrosis factor-α (TNF-α)-dependent hepatic apoptosis induced by D-galactosamine (D-GalN) and lipopolysaccharide (LPS) in mice.

Materials and methods

Chemicals

D-GalN and collagenase were purchased from Wako Pure Chemicals Industry (Osaka, Japan) and LPS (Escherichia coli 055:B5) was from Difco Laboratories (Detroit, MI, USA). Mouse recombinant TNF-α, silymarin, William's E medium, bovine serum albumin (BSA), insulin, dexamethasone, penicillin G, streptomycin and 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT) were purchased from Sigma Chemicals (St. Louis, MO, USA). Ethylene glycol-*O*,*O*′-bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) was purchased from Fluca Chemie (Switzerland) and heat-inactivated calf serum and Hanks' balanced salt solution (HBSS) were from Gibco BRL Products (Gaitherburg, MD, USA). Falcon primaria surface-modified polystyrene culture plates with 96 wells were from Becton Dickinson (Lincoln Park, NJ, USA). Serum glutamic pyruvic transaminase (sGPT) and serum glutamic oxaloacetic transaminase (sGOT) activities were measured with a Transaminase CII-Test kit (Wako Pure Chemicals Industry, Osaka, Japan). Serum TNF-α and IFN-γ concentrations were determined using an enzyme-linked immunosorbent assay (ELISA) with anti-mouse TNF-α antibody and a Mouse IFN-γ ELISA kit (Endogen, Inc., USA), respectively.

Isolation of secoisolariciresinol (1) and isotaxiresinol (2)

Dried wood of *T. yunnanensis* (850 g) was treated with H_2O (4 l × 3) under reflux for 30 min to yield an H_2O extract (52.2 g). The extract was then divided into EtOAc soluble (34.1 g) and insoluble (16.1 g) fractions. The EtOAc soluble fraction (32.0 g) was subjected to chromatography on a silica gel column (60 × 3.5 cm) and eluted with MeOH/$CHCl_3$ (0–30%) to give nine fractions (each 500 ml) of eluate. Secoisolariciresinol (**1**, 3.62 g) was obtained from fractions 4 and 5 (1–5% MeOH/$CHCl_3$ eluates) by fractional crystallization, while fraction 7 (10–20% MeOH/$CHCl_3$ eluates) gave isotaxiresinol (**2**, 7.84 g). Their physical and spectral data were found to be identical with those reported in the literature (Agrawal and Rastogi, 1982; King et al., 1952).

Animals

Male ddY mice, 6 weeks old (30–32 g), were used for the D-GalN/LPS-induced liver injury model. All the animals were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan) and maintained on a 12 h light/dark cycle in a temperature and humidity controlled room. The animals were given a laboratory pellet chow (CE-2; CLEA Japan Inc., Tokyo, Japan) and water ad libitum. This study was conducted in accordance with the standards outlined in the Guidelines for the Care and Use of Laboratory Animals of Toyama Medical and Pharmaceutical University, Toyama, Japan.

D-GalN/LPS-induced liver injury in mice

Liver injury was induced in 12-h fasted mice by intraperitoneal (*i.p.*) injection of D-GalN (700 mg/kg) and LPS (10 μg/kg) as described previously (Tiegs, 1994). Lignans **1** and **2** were injected *i.p.* at 50 and 10 mg/kg twice 12 and 1 h before the D-GalN/LPS administration. Saline was given to control mice. The group of mice treated twice with silymarin (100 mg/kg, p.o.) before the D-GalN/LPS injection was used as a positive control group. Blood for TNF-α and IFN-γ measurements was sampled from the venous plexus of the ocular fundus under ether anesthesia at 90 min after the D-GalN/LPS administration. Blood for the serum transaminase measurement was collected in the same way at 8 h after the administration. The liver was collected simultaneously. One lobe was kept in liquid nitrogen for DNA fragmentation analysis and the other was immersed in 10% neutralized formalin solution for histological analysis. Blood of mice that died within 8 h was withdrawn by cardiac puncture.

Histology

The liver, fixed in a formalin solution, was embedded in paraffin, sectioned at 4-μm, stained with hematoxylin-eosin and photographed at a magnification of ×400. Apoptotic bodies and nuclei displaying chromatin condensation were observed and compared between each group.

Liver DNA extraction and detection of DNA fragmentation

The frozen liver (ca. 200 mg) was ground and suspended in ice-cold phosphate-buffered saline. The suspension was centrifuged at 1,500 rpm for 5 min. The cell pellet was suspended in 1 ml of lysis buffer (10 mM Tris-HCl buffer, pH 7.5, 10 mM EDTA, and 0.2% Triton X-100) and kept on ice for 10 min. The lysate was centrifuged at 14,000 rpm for 10 min and 400 μl of supernatant was collected. After TE (10 mM Tris-HCl, pH 7.5; 1 mM EDTA, pH 7.5)-saturated phenol (Wako Pure Chemical Industries, Osaka, Japan) was added, the mixture was vortexed and then centrifuged at 14,000 rpm for 10 min. The upper layer was collected and mixed with an equal volume of CIAA solution (chloroform:isoamyl alcohol, 24:1). DNA in the upper aqueous phase was precipitated with 3 M NaCl and cold ethanol after an overnight incubation at −20°C. After drying, the DNA pellet was dissolved in TE buffer and incubated with 10 μg/ml of RNase (sigma Chemicals, Saint Louis, MO, USA) at 37°C for 30 min. Following the addition of loading buffer (Wako Pure Chemical Industries, Osaka, Japan), fragmented DNA was separated by 1.5% agarose gel electrophoresis at 100 V and visualized by staining with 100 ng/ml of ethidium bromide (Wako Pure Chemical Industries, Osaka, Japan).

TNF-α-induced cell death in primary cultured mouse hepatocytes and murine L929 fibrosarcoma cells

Mouse liver parenchymal cells were isolated according to the procedure described by Seglen (1976). In brief, the liver was perfused with Ca^{2+}-free HBSS containing 0.5% BSA and 5 mM EGTA, then re-circulated with a collagenase solution composed of Ca^{2+}-free HBSS, 0.075% collagenase, 4 mM $CaCl_2$, and 0.005% trypsin inhibitor. Isolated hepatocytes were cultured in William's E medium supplemented with 10% calf serum, 100 IU/ml of penicillin G, 100 μg/ml of streptomycin, 100 μM dexamethasone and 50 ng/ml of insulin and incubated in 96-well plastic plates (1.5×10^4 cells/well). After 2 h pre-incubation, the medium was replaced with fresh medium containing D-GalN (0.5 mM) and test

specimens at various concentrations. Thirty minutes later, TNF-α (100 ng/ml) was added to each well, and the viability of the hepatocytes was assessed 18 h thereafter by determination of the MTT colorimetric reaction.

The L929 cell line purchased from Riken Cell Bank (Tsukuba, Japan) was cultured in EMEM medium supplemented with 0.1% sodium bicarbonate and 2 mM glutamine, penicillin G (50 units/mL), streptomycin (50 μg/mL), 0.1 mM nonessential amino acids and 10% fetal calf serum. The cells were harvested with trypsin and diluted in fresh medium. The experiment was performed according to a previously reported procedure with some modification (Hehner et al., 1998). In brief, the cells were seeded in 96-well plastic plates with 1×10^4cells/well and allowed to adhere for 2 h at 37°C in a humidified atmosphere containing 5% CO_2. Then the medium was replaced with fresh medium, containing TNF-α (10 ng/mL) together with test compounds at various concentrations and the cells were incubated for 24 h. Cellular viability was determined using the standard MTT assay.

Statistics

Statistical analyses were performed using the unpaired Student *t*-test. Data are expressed as the mean ± SD of four determinations for the in vitro experiments, and the mean ± SE for in vivo experiments. A significant difference was accepted at $p < 0.05$.

Results

Co-administration of D-GalN (700 mg/kg) and LPS (10 μg/kg) to mice results in a typical hepatic apoptosis characterized by DNA fragmentation, chromatin condensation, and the formation of intracellular apoptotic bodies. The hepatic DNA extracted from the liver of the mice at 8 h after the D-GalN/LPS treatment exhibited a typical ladder of DNA fragmentation upon electrophoresis in a 1% agarose gel (Fig. 2). The *i.p.* administration of **1** and **2** (50 mg/kg) and sylimarin (100 mg/kg, *p.o.*) 12 and 1 h before

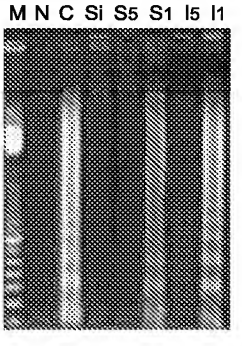

Fig. 2. Electrophoresis of hepatic DNA. The DNA was extracted from mouse liver at 8 h after the d-GalN/LPS injection. N: untreated normal, C: D-GalN/LPS-treated control, S5: D-GalN/LPS + **1** (50 mg/kg), S1: D-GalN/LPS + **1** (10 mg/kg), I5: D-GalN/LPS + **2** (50 mg/kg), I1: D-GalN/LPS + **2** (10 mg/kg), Si: D-GalN/LPS + silymarin (100 mg/kg, *p.o.*), M: 100 bp ladder marker.

the D-GalN/LPS treatment reduced the extent of DNA fragmentation (Fig. 2). A histological examination of the liver specimens showed the appearance of large numbers of apoptotic bodies and nuclei with condensed chromatin in the D-GalN/LPS-treated control group (Fig. 3B). In the mice administered **1** and **2** at 50 and 10 mg/kg (*i.p.*), chromatin condensation and apoptotic body formation were found to be inhibited (Fig. 3C–3F) to a similar extent to that in the silymarin (100 mg/kg, *p.o.*)-treated positive control group.

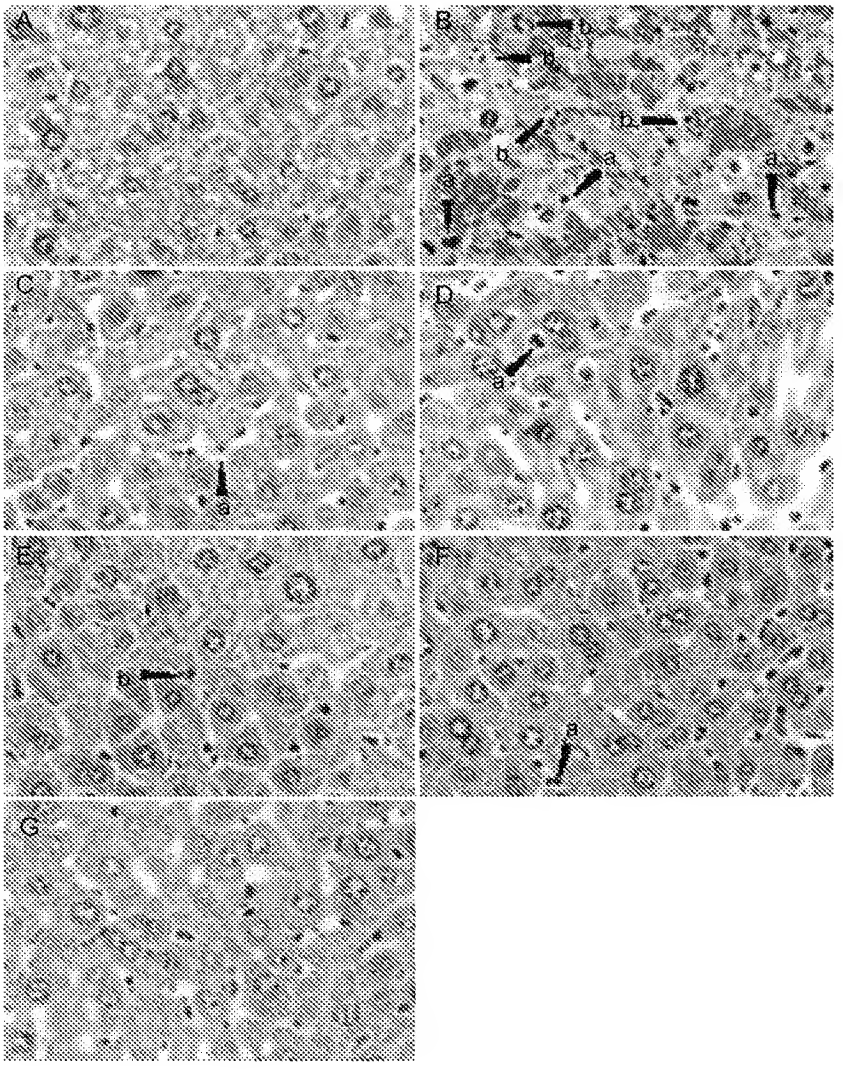

Fig. 3. Histopathlogy of the liver (× 400). Liver samples were collected 8 h after the D-GalN/LPS injection. A) Normal mice without any treatment, B) D-GalN/LPS-treated control, C) D-GalN/LPS + **1** (50 mg/kg), D) D-GalN/LPS + **1** (10 mg/kg), E) D-GalN/LPS + **2** (50 mg/kg), F) D-GalN/LPS + **2** (10 mg/kg), G) D-GalN/LPS + silymarin (100 mg/kg, *p.o.*). Apoptosis is manifested by intracellular apoptotic bodies (a) and nuclei with chromatin condensation (b).

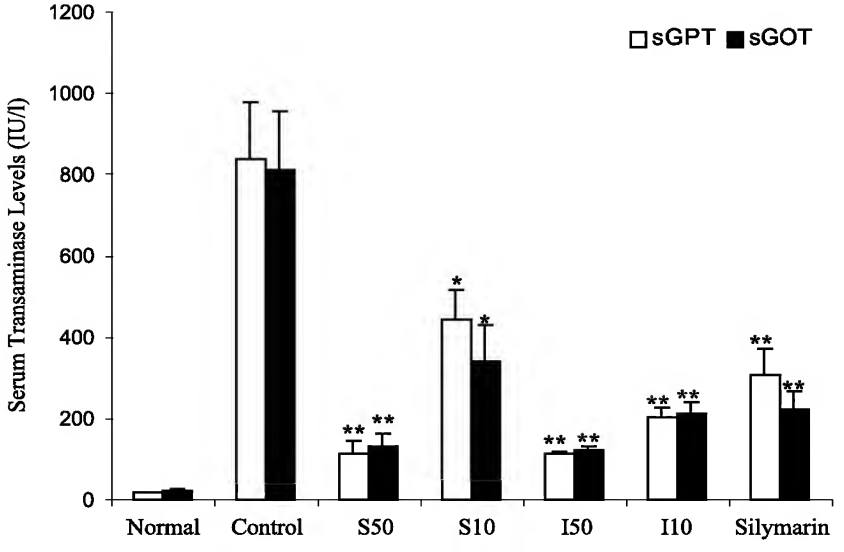

Fig. 4. Inhibition by *i.p.* administration of secoisolarisiresinol (**1**) and isotaxiresinol (**2**) of increases in sGPT and sGOT in D-GalN/LPS-intoxicated mice. Mice were administered **1**, **2** or saline (for control animals) 12 h and 1 h before the D-GalN/LPS injection. Blood was collected 8 h after the injection. Normal: mice without any treatment, Control: D-GalN/LPS, S50: D-GalN/LPS + **1** (50 mg/kg), S10: D-GalN/LPS + **1** (10 mg/kg), I50: D-GalN/LPS + **2** (50 mg/kg), I10: D-GalN/LPS + **2** (10 mg/kg), Silymarin: D-GalN/LPS + silymarin (100 mg/kg, *p.o.*). Data expressed as means ± SE. ($n = 6$; for normal, $n = 3$). * $p < 0.05$, ** $p < 0.01$, significantly different from the D-GalN/LPS-treated control.

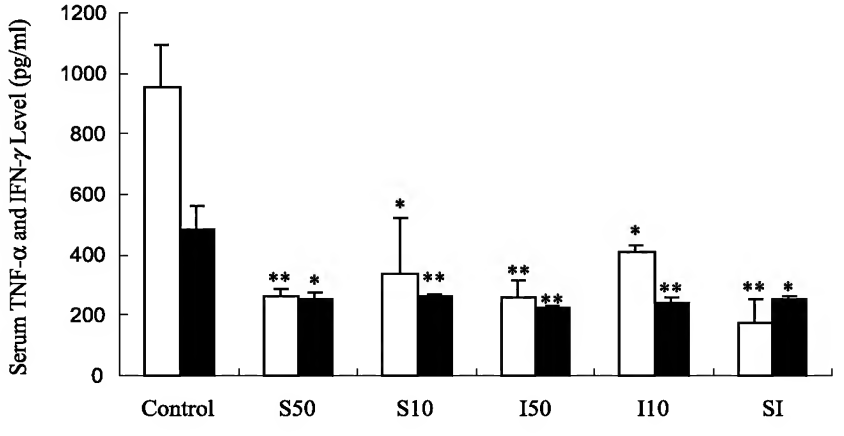

Fig. 5. Effect of secoisolarisiresinol (**1**) and isotaxiresinol (**2**) on serum TNF-α and IFN-γ concentrations at 90 min after the D-GalN/LPS injection. Control: D-GalN/LPS-treated control, S50: D-GalN/LPS + **1** (50 mg/kg), S10: D-GalN/LPS + **1** (10 mg/kg), I50: D-GalN/LPS + **2** (50 mg/kg), I10: D-GalN/LPS + **2** (10 mg/kg), SI: D-GalN/LPS + silymarin (100 mg/kg, p.o.). Serum TNF-α levels in untreated normal mice were below the detectable limit (< 10 pg/mL), while IFN-γ levels of normal mice were 226.3 ± 7.5 pg/ml. Data are expressed as the mean ± SE ($n = 6$; for normal, $n = 3$). * $p < 0.05$, ** $p < 0.01$, significantly different from control.

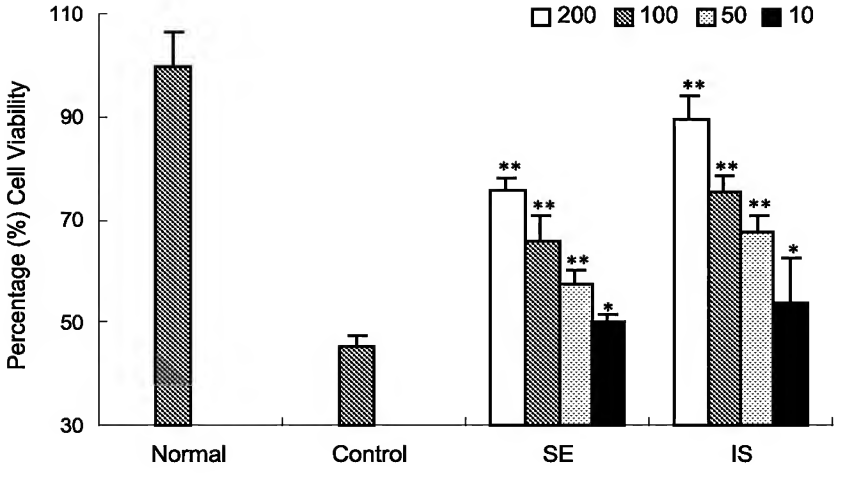

Fig. 6. Protective effect of secoisolarisiresinol (**1**) and isotaxiresinol (**2**) on D-GalN/TNF-α-induced cell death in primary cultured mouse hepatocytes. Viability of hepatocytes (1.5×10^4 cells) in plastic 96-well plates at 18 h after TNF-α addition, with or without **1** and **2**. Results are expressed as the mean ± SD ($n = 8$). Normal: untreated mice, Control: D-GalN/TNF-α-treated control, SE: **1**, IS: **2**, 200, 100, 50 and 10: D-GalN/TNF-α + **1** or **2** at 200, 100, 50, and 10 μM. * $p < 0.01$, ** $p < 0.001$ significantly different from control.

sGPT and sGOT concentrations were increased to 837.7 ± 140.9 and 811.1 ± 142.7 IU/L, respectively, at 8 h after the D-GalN/LPS injection. sGPT and sGOT levels in normal mice without D-GalN/LPS treatment were 16.1 ± 0.5 and 24.5 ± 2.0 IU/L, respectively. Pre-administration of **1** and **2** before the injection of D-GalN/LPS significantly reduced these pathological events in a dose-dependent manner (Fig. 4).

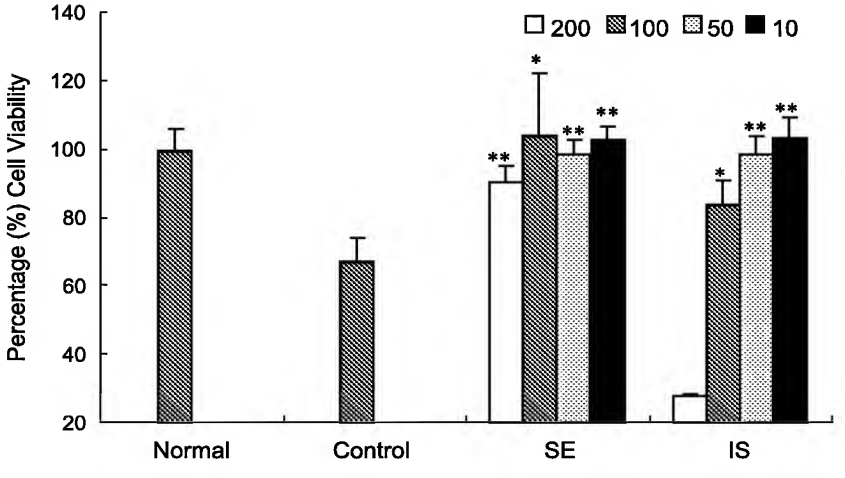

Fig. 7. Protective effect of secoisolarisiresinol (**1**) and isotaxiresinol (**2**) on TNF-α-induced toxicity in murine L929 fibrosarcoma cells. Viability of L929 cells (1×10^4) in 96-well plates at 24 h after TNF-α addition, with or without **1** and **2**. Results are expressed as the mean ± SD ($n = 8$). Normal: untreated mice, Control: TNF-α-treated control, SE: **1**, IS: **2**, 200, 100, 50 and 10: TNF-α + **1** or **2** at 200, 100, 50, and 10 μM. * $p < 0.01$, ** $p < 0.001$ significantly different from control.

We further measured serum TNF-α and IFN-γ levels 90 min after the D-GalN/LPS injection, to know whether **1** and **2** prevent the production of proinflammatory cytokines considered to be important factors. Both TNF-α and IFN-γ levels were found to be significantly inhibited by treatment with **1** and **2** at 10 and 50 mg/kg (Fig. 5).

The protective effect of **1** and **2** on D-GalN/TNF-α-induced cell death in primary cultured mouse hepatocytes and TNF-α-mediated toxicity in L929 cells were also tested in vitro. Both **1** and **2** protected hepatocytes from TNF-α-induced cell death significantly and dose-dependently (Fig. 6). The cell survival rates of secoisolariciresinol (**1**)-treated groups were 75.6 ± 2.5, 65.9 ± 4.9, 57.9 ± 2.2 and 50.2 ± 1.5%, at concentrations of 200, 10, 50 and 10 μg/ml, respectively. The cell survival rates of the isotaxiresinol (**2**)-treated groups were 89.6 ± 4.2, 75.7 ± 2.8, 68.1 ± 2.6 and 54.0 ± 8.6%, respectively, at the same concentrations. Treatment with these lignans at various concentrations further protected L929 cells from TNF-α-induced toxicity: one exception was isotaxiresinol (**2**) at 200 μM (Fig. 7). These results indicate that **1** and **2** protect hepatocytes from apoptosis mediated by TNF-α.

Discussion

D-GalN/LPS-induced liver failure in mice is a promising animal model for elucidating the mechanisms of clinical liver complaints and for evaluating the efficiency of hepatoprotective activity (Tiegs, 1994; Hase et al., 2001; Matsuda et al., 2001). TNF-α plays a central role in the pathogenesis of D-GalN/LPS-induced liver injury in mice, though other inflammatory cytokines such as IFN-γ and free radicals also contribute. LPS stimulates macrophages to secrete various pro-inflammatory cytokines, including interleukin-1 (IL-1), IL-6, IFN-γ and considerable amounts of TNF-α (Martich et al., 1991). TNF-α induces apoptosis in hepatocytes and neutrophil transmigration, a critical step in the necrosis of hepatocytes, occurs at a later stage in this liver injury (Tiegs et al., 1989; Chosay et al., 1997). The hepatic lesions in this model resemble those of human hepatitis because the up-regulation of TNF-α expression and hepatic apoptosis have been reported as pathogenic symptoms in human hepatitis. Using this animal model, we investigated various medicinal plants and isolated tetrahydroswertianolin (THS), acteoside and majonoside R_2 from *Swertia japonica*, *Cistanche deserticola* and *Panax vietnamensis*, respectively, as potent hepatoprotective agents (Hase et al., 1999; Xiong et al., 1999; Tran et al., 2002). These active compounds significantly inhibited the D-GalN/LPS-induced apoptosis of hepatocytes via different mechanisms.

Phytochemical investigation of the wood of *Taxus yunnanensis* led to the isolation of secoisolariciresinol (**1**, yield 0.6826%) and isotaxiresinol (**2**, yield 0.9777%) as major components (Banskota et al., 2002, 2003). In the present study, we investigated the effect of **1** and **2** on hepatic apoptosis in a D-GalN/LPS-induced liver injury model. Fragmentation of DNA and the formation of apoptotic bodies, which are respectively biochemical and morphological hallmarks of apoptotic cell death, act to initiate D-GalN/LPS-induced liver injury. Previously, we demonstrated that hepatic DNA fragmentation increased 2123% with respect to the value in untreated mice (set at 100%) at 5 h after D-GalN/LPS treatment without a significant elevation in serum transaminase (Hase et al., 1999). Electrophoresis of the hepatic DNA at 8 h after D-GalN/LPS injection showed a typical ladder of oligonucleosomal DNA fragmentation, which was significantly inhibited by pretreatment with **1** and **2** at a dose of 50 mg/kg 12 and 1 h before the D-GalN/LPS injection (Fig. 2). In the histological examination, numerous apoptotic bodies and extensive chromatin condensation were observed in the liver of D-GalN/LPS-treated (Fig. 3B)

control mice. Pre-treatment with **1** and **2** at 50 and 10 mg/kg significantly reduced the number of apoptotic bodies and degree of chromatin condensation (Fig. 3C–F) suggesting that **1** and **2** inhibited the hepatic apoptosis induced by D-GalN/LPS. Moreover, the sGPT and sGOT levels, which were increased to 837.7 ± 140.9 and 811.1 ± 142.7 IU/L, respectively, due to the results of a severe necrosis in the liver 8 h after D-GalN/LPS treatment, were significantly and dose-dependently lowered by pretreatment with **1** and **2** (Fig. 4). These results led us to conclude that both **1** and **2** prevented hepatic apoptosis as well as the subsequent necrotic liver damage. The protective effect of these lignans was found to be comparable to that in the silymarin (100 mg/kg, *p.o.*)-treated positive control group. To the best of our knowledge, this is the first report that these two lignans (**1** and **2**) have hepatoprotective activity.

As mentioned above, TNF-α secreted from LPS-activated macrophages induces the apoptosis of hepatocytes as well as the transmigration of neutrophils critical to the necrosis of hepatocytes which occurs at a later stage of this liver injury. Thus, a compound which inhibits TNF-α production may suppress the pathophysiology in the D-GalN/LPS-induced liver injury model (Hase et al., 1999; Tran et al., 2002; Matsuda et al., 2002). In order to clarity whether pre-treatment with **1** and **2** blocks the production of TNF-α, we measured the serum TNF-α level 90 min after D-GalN/LPS injection. Both **1** and **2** inhibited the production of TNF-α in a significant and dose-dependent manner (Fig. 5). We also examined the IFN-γ level in serum. The IFN-γ levels of mice pretreated with **1** and **2** were significantly decreased compared to those of the D-GalN/LPS-treated control group (Fig. 5). There was a good correlation between the inhibition of pro-inflammatory cytokines TNF-α and IFN-γ and the decreases in sGPT and sGOT levels of the mice treated with **1** and **2**, suggesting that **1** and **2** prevent hepatocyte apoptosis by inhibiting TNF-α as well as IFN-γ production in LPS-activated macrophages.

We further tested the protective effect of **1** and **2** on D-GalN/TNF-α-induced cell death in primary cultured mouse hepatocytes and TNF-α-mediated toxicity in murine L929 fibrosarcoma cells in order to elucidate their influence on the apoptosis regulated by TNF-α. Both lignans significantly inhibited but did not completely block the production of TNF-α and IFN-γ measured at 90 min after intoxication (Fig. 5). In D-GalN/TNF-α-induced cell death, apoptosis was reported to occur early from 8 h and to last to 20 h, while necrosis occurred later at 16 h after the D-GalN/TNF-α-treatment (Leist et al., 1994). Both **1** and **2** at 200, 100, 50 and 10 μM significantly attenuated the cell death induced by D-GalN/TNF-α and the effect was concentration-dependent (Fig. 6). Moreover, **1** and **2** protected L929 cells from the toxicity of TNF-α (Fig. 7). These results suggested that both **1** and **2** prevent the TNF-α-induced apoptosis of hepatocytes. TNF-α-dependent apoptosis is a complicated process but it has been well documented that TNF-α generates reactive oxygen species (ROS) by the uncoupling of mitrochondrial respiration, by the activation of xanthine oxidase and by the lipopoxygenus pathway (Cossarizza et al., 1997; Larrick and Wright, 1990; O'donnell et al., 1995). The ROS then activate the transcriptional factor NF-κB as well as phospholipase and endonuclease, leading to apoptotic cell death (Larrick and Wright, 1990; Marinovich et al., 1996). It has also been reported that TNF-α induces oxidative stress specifically in isolated mouse hepatocytes (Adamson and Billings, 1992). Moreover, lignans **1** and **2** both possessed strong DPPH radical scavenging activity and significant inhibitory activity against LPS-activated NO production in murine macrophage-like J774.1 cells (Banskota et al., 2003). All these results led us to conclude that the antioxidative properties of these lignans contribute to the inhibition of TNF-α-mediated cell death, even though mechanisms such as attenuation of the inhibition of protein synthesis induced by D-GalN and inhibition of TNF-α-receptor activation of caspase 8 may also be involved (Reutter and Hassels, 1980; Kawaguchi et al., 1999; Jones et al., 2000).

In conclusion, the present report shows that secoisolariciresinol (**1**) and isotaxiresinol (**2**), major lignans isolated from the wood of *T. yunnanensis*, protect hepatocytes from apoptosis by inhibiting the production of TNF-α and IFN-γ by LPS-activated macrophages and directly inhibit the apoptosis induced by TNF-α. The present study supports the possible use of phytoestrogens especially plant-derived dibenzylbutane and aryltetralin lignans for the treatment of hepatitis and other liver complications though further study is needed.

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